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14. ABSTRACT

Androgens, mediated by the Androgen Receptor (AR), play a crucial role in prostate cancer. Current treatments include antiandrogens competing with natural androgens and antagonize AR transcriptional activity. Although widely used, antiandrogens have shown significant side effects. Regulation of gene expression by AR requires the binding to its natural ligand, dihydrotestosterone (DHT), and assembly of coregulatory proteins (CoR). The blockage of the interaction between DHT-liganded AR and CoR by small molecules has been shown to inhibit gene transcription. Targeting this interaction might be a relevant way to regulate prostate cancer proliferation. Preliminary data revealed that flufenamic acid (FLF) binds to AR and inhibit the recruitment of CoR. We have synthesized a series of FLF analogs, evaluated their solubility, cell permeability and cytotoxicity profiles. We report the biochemical activity of these compounds towards their ability to block CoR binding. In cell based assays, we show the bioactivity of these inhibitors at a gene transcription level. Thus, we provide the first class of small molecules able to inhibit AR transcription activity. Additionally, we optimized an AR scintillation proximity (SPA) competition ligand binding assay. We showed that this assay can be used to measure ligand affinities for a range of nuclear receptors.

15. SUBJECT TERMS
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Introduction

The Androgen Receptor (AR) is a ligand-dependent transcription factor that belongs to the nuclear hormone receptor superfamily. Once the allosteric site of AR recognizes an androgen (i.e. dihydrotestosterone (DHT)), it undergoes a conformational rearrangement. The receptor then becomes active by revealing activation pockets on its surface in the ligand-binding domain (LBD) that is necessary for the recruitment of coregulatory proteins (CoR) involved in the gene transcription. After the binding of these CoR, a number of accessory and intermediary proteins (Androgen Response Elements) associate with the complex allowing reformatting of the chromosomal DNA and regulation of gene transcription. Because prostate cancer cell proliferation is often androgen-dependent, androgen pathway blockage is the standard therapy for the treatment of prostate cancer. However, current antiandrogens, like flutamide (Drogenil), have strong side effects (suppression of masculinity, osteoporosis…). In addition tumors have become resistant suggesting mutations of the receptor.
As CoR recruitment is a critical step for AR-dependent gene transcriptional activation, targeting this site might be a relevant way to regulate prostate cancer proliferation. Moreover, this innovative inhibition strategy should overcome the drawbacks posed by therapies (antiandrogen resistance, side effects) currently applied in prostate cancer treatment.
Preliminary data revealed that the non-steroidal anti-inflammatory drug flufenamic acid (FLF) binds to the AR and inhibits the recruitment of CoR. Herein we describe the development of small molecules, analogues of FLF, using biochemical and cellular assays to establish careful structure activity relationship (SAR) models. Small molecule inhibitors of the interaction between liganded AR and CoR represent powerful assets to study the mechanism of AR transcriptional function and a new potential therapeutic modality for prostate cancer.

Body

Task I. Development of Focused Libraries for Optimization of AR Co-Regulator Interaction Inhibitors.

Our experimental approach for finding compounds that bind to the AR-LBD and compete with CoR utilized high throughput screening (HTS) based on a biochemical assay [1, 2]. We screened a highly diversified collection of about 55,000 drugs using a HTS competition assay based on fluorescence polarization technique, which we developed for monitoring displacement of NR box probes from liganded NR-LBD [2, 3]. This very specific method identified five compounds that blocked fluorescently labeled peptide recruitment at the surface of the DHT-liganded AR. This preliminary study was confirmed by robust secondary biochemical assays and provided us flufenamic acid (FLF, Fig. 1A), a non-steroidal anti-inflammatory drug (NSAID), as a hit compound. Unexpectedly, crystallography studies revealed that FLF is not binding to AF2, a well described transcriptional activation function domain onto the LBD [4, 5]; but is binding at a secondary external site named BF3 (Figure 1B and 1C)[3]. BF3 is a second ligand-induced hydrophobic cleft at the surface of AR, allosteric regulator of AF2. Thus, FLF binding in BF3 induces structural rearrangements that are propagated to AF2, causing the alteration of AF2 conformation that consequently blocks the CoR assembly and gene transcription.

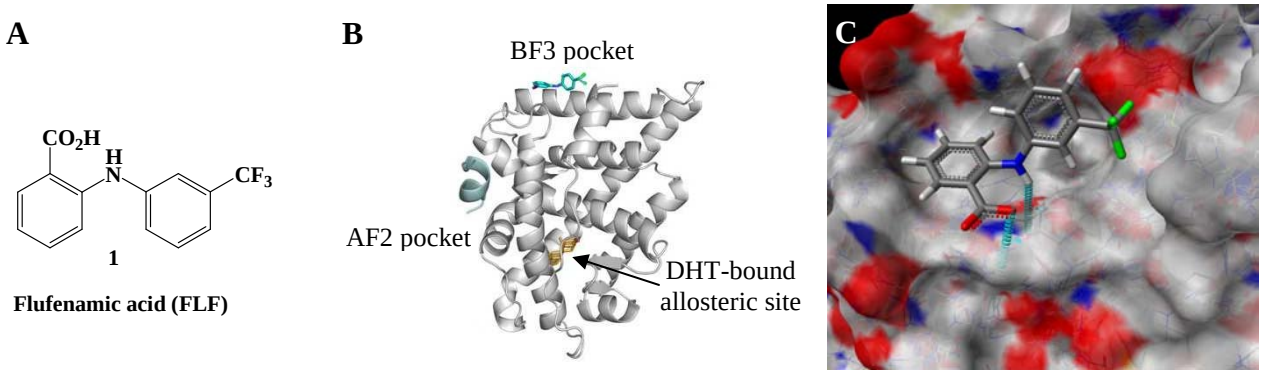


Figure 1. A. Chemical structure of flufenamic acid (FLF), hit compound identified by fluorescence polarization HTS assay[3]. **B.** Schematic view of AR LBD showing DHT-bound allosteric site (DHT shown in orange stick models), the location of the CoR active binding site (AF2) and the second external binding site (BF3). Depicted in blue stick models is flufenamic acid bound to BF3. The figure was generated with Pymol. **C.** Crystal structure of FLF binding to BF3 pocket.

I.1. Drug Design. We designed novel scaffolds based on the structure of flufenamic acid, the relevant NSAID derivative provided by the HTS campaign. We were able to build SAR models and correlate the features of actives compounds to choose scaffolds that accommodate the consensus features. We based our design on a crystal structure of FLF binding to BF3 at the surface of DHT-liganded AR (Fig. 1C) [3]. Several structural aspects appeared to be crucial from this study. First, the carboxylic group seemed to be required, presumably due to interaction with a glycine residue (G724, 2.9 Å) on the periphery of the binding pocket. Second, the secondary amine moiety appeared to enhance the affinity of the small molecule via its binding to a proline residue (P723, 3.4 Å). Third, the crystal structures of our initial hits revealed that several of them overlap to fill, in aggregate, the entire binding pocket [3]. Thus, one might hypothesize that a higher affinity compound could be produced with a "V-shape" scaffold. Finally, looking at the shape of the BF3 hydrophobic external pocket, we could easily envision that the conformation of the compound allowed free-space located around the second aromatic ring (the first ring is bearing the acid function). To explore those hypotheses, we used different individual compounds and studied a focused library.

The first variation study focused on the acid function. To address the importance of the free carboxylic acid group, the corresponding methyl ester **2** was prepared (Scheme 1). Then, in order to evaluate the potential binding of the aryl amine to the proline residue, we directly replaced the secondary amine either by an ether (**3**) or a thioether (**4**) moiety (Scheme 1). Following the same purpose, we elongated the spacer function between the two aromatic rings by introducing an additional methylene group (**5** and **6**) or a carbonyl group (**7**) (Scheme 1). Several tricyclic analogs of FLF were synthesized to test if rigid structures would improve the fitting to the BF3 pocket (Scheme 2). At the same time, we explored the potential for more effectively filling the binding pocket by addition of moieties to the second aromatic ring of the diarylamine. A focused library was prepared to give better understanding of what substituents are tolerated and how they affect the binding mode of the small molecule (Scheme 3).

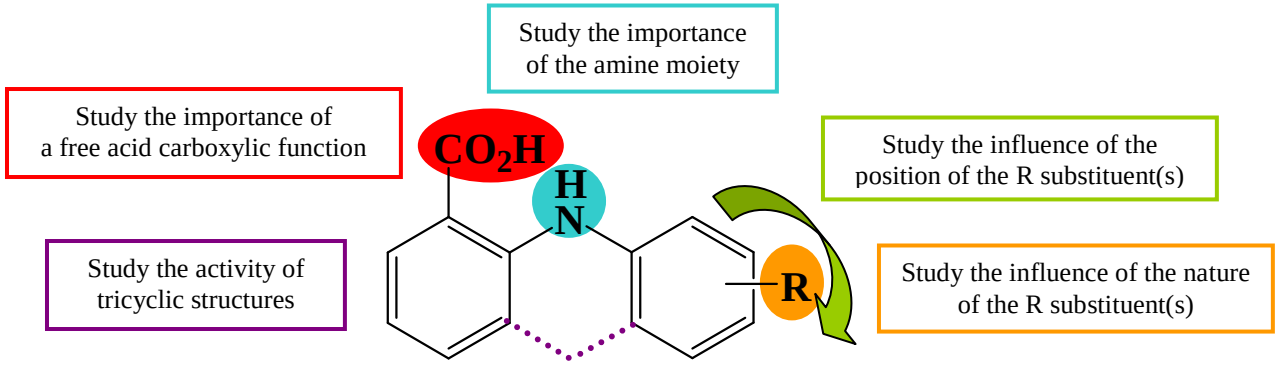
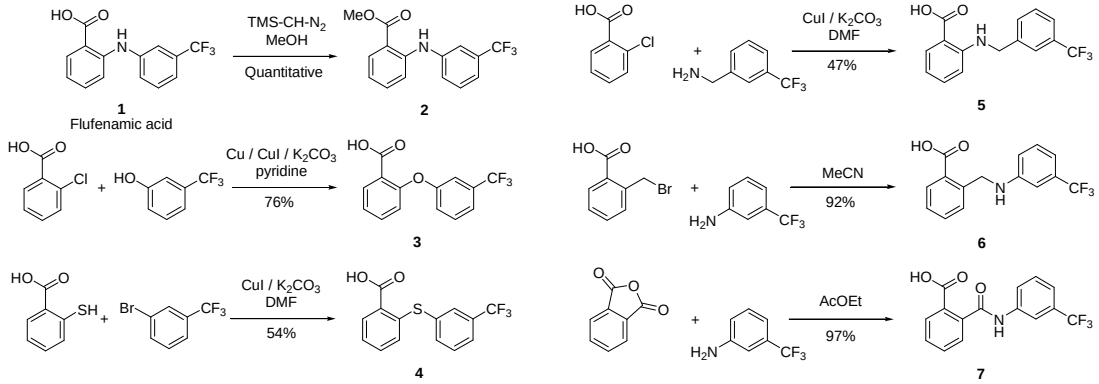


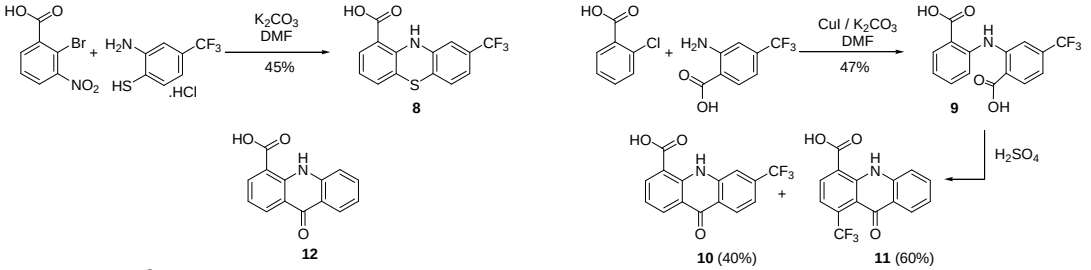
Figure 2. Schematic summary of the structural modifications performed on flufenamic acid scaffold.

I.2. Drug Synthesis. We then synthesized small non-peptide molecules after optimizing diverse synthetic strategies. We adapted synthetic pathways to parallel chemistry techniques and purified compounds using automated methods. The methyl ester **2** was quantitatively obtained by treatment of the commercially available flufenamic acid **1** with (trimethylsilyl)diazomethane (Scheme 1). The ether **3**, the thioether **4** and the derivative **5** were synthesized using variants of the Ullman coupling with copper iodide. Nucleophilic attack of (trifluoromethyl)aniline either on 2-bromomethylbenzoic acid or on the phthalic anhydride gave respectively compounds **6** and **7** with high yields.



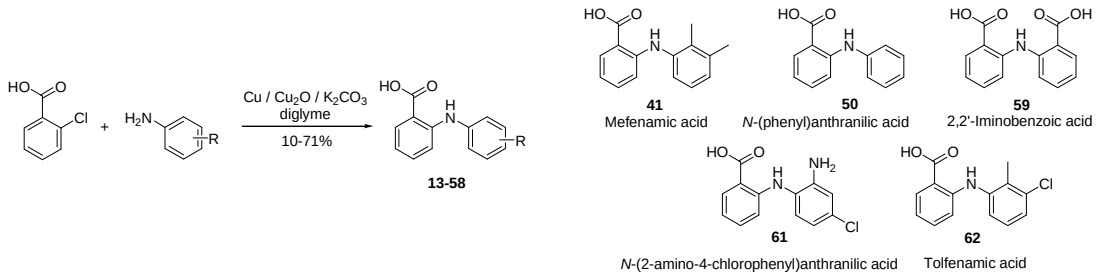
Scheme 1. Variations on the carboxylic and aryl amine functions of flufenamic acid.

Tricyclic compound **8** was synthesized according to literature procedure [6] (scheme 2). A Ullman coupling was performed with 2-chloroanthranilic acid and 3-(trifluoromethyl)-6-carboxy-aniline to give the diacid **9**. By intramolecular cyclisation under acidic conditions, the isomers **10** and **11** were obtained in a ratio of 2:3. The commercially available 4-carboxy-9-acridone (**12**) was also tested.



Scheme 2. Synthesis of tricyclic FLF derivatives.

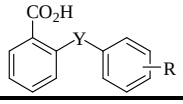
Finally a focused library of 45 substituted FLF analogs was obtained in moderate yields (see table 1) by reaction of the 2-chlorobenzoic acid and either commercially available or made-in-house anilines. Commercially available anthranilic derivatives (**59-63**) were added to this collection of FLF derivatives to be tested.



Scheme 3. General synthesis scheme of FLF analogs and structures of commercially available FLF derivatives.

I.3. Quality Control. Each new compound was submitted to a strict quality control. All structures were confirmed by nuclear magnetic resonance spectroscopy (^{1}H NMR, ^{13}C NMR), UV and IR spectroscopy. Purity of each compound was evaluated by LC-MS.

I.4. Binding Affinities of Coregulator Interaction Inhibitors to the Androgen Receptor. To monitor if small molecules are able to displace a labeled peptide mimicking the NR box from the AF2 site *via* binding to the AR surface, we developed a FP assay [3]. Conceptually, the binding of a fluorescent molecule to another molecule can be quantified by the change in its speed of rotation. In the present case, free fluorescent CoR peptide rotates more rapidly and has a lower FP value than the FP value corresponding to the complex AR-fluorescent peptide. Fluorescently labeled SRC2-3 peptide and DHT liganded AR-LBD protein were incubated with varying concentrations of compound for 5 hours. Because the measured polarization is an average of the free and bound fluorescent CoR peptide, it can be used to assess competitive displacement from the binding site and to establish dose-response curve analysis, and determine IC_{50} values. Binding affinities were evaluated for each compound using a competitive fluorescence polarization assay in three independent experiments (table 1). This binding assay meets the Bland-Altman quality and reproducibility criteria [7] with an overall minimum significant ratio (MSR < 3) of 2.85.

Cpd					Yield (%)	SRC2-3 competition (FP assay)	DHT competition (SPA assay)
	Y	R					
		o	*m*	*p*		IC_{50} (µM)	IC_{50} (µM)
2	NH	*Methyl ester derivative, see scheme 1*			100	-	-
3	O	CF_3				-	-
4	S	CF_3				-	-
5	NH-CH_2	CF_3			>500	-	-
6	CH_2-NH	CF_3				-	-
7	CO-NH	CF_3				-	-
8	NH	*Tricyclic compound see scheme 2*			45	-	67 ± 42
9	NH	CF_3, CO_2H			>500	-	-
10	NH	*Tricyclic compound see scheme 2*			60	-	-
11	NH	*Tricyclic compound see scheme 2*			40	-	10 ± 6
12	NH	*Tricyclic compound see scheme 2*			com.	-	43 ± 10
13	NH			*cy*-Hex	38	46.5 ± 8	4.4 ± 0.5
14	NH			SPh	11	16 ± 4	3.0 ± 0.2
15	NH	SPh			21	104 ± 25	50 ± 18
16	NH		di-OPh		23	19 ± 5	57 ± 54
17	NH			OPh	34	27.5 ± 5	7.4 ± 1.1
18	NH		OPh		18	41 ± 20	122 ± 97
19	NH	OPh			34	48 ± 28	-
20	NH			Bn	15	17 ± 8	5.8 ± 1.4
21	NH		Bn		29	56 ± 15	101 ± 87
22	NH	Bn			19	54 ± 13	281 ± 249
23	NH			Ph	10	33 ± 12	19.4 ± 9.2
24	NH		Ph		33	80 ± 24	71 ± 36
25	NH	Ph			10	93 ± 25	-
26	NH		Ph		19	75 ± 16	-
27	NH			*t*Bu	36	64 ± 20	
28	NH		*t*Bu		45	123 ± 37	269 ± 232
29	NH		OBn		41	30 ± 9	
30	NH	OBn			71	70 ± 23	87 ± 66
31	NH			*n*Hex	10	39 ± 20	4.4 ± 0.7
32	NH			O-*n*Hex	24	-	4.4 ± 1.2
33	NH			OPyridin	33	-	37 ± 13
34	NH		OPyridin		13	-	-
35	NH			*i*Pr	22	133 ± 54	-
36	NH		*i*Pr		38	235 ± 90	-

37	NH	*i*Pr			10	110 ± 32	-
38	NH			CF_3	18	39 ± 13	-
1	NH		CF_3		com.	102 ± 39	-
39	NH	CF_3			12	-	-
40	NH			SCF_3	10	168 ± 73	41 ± 17
41	NH	Me	Me		com.	112 ± 26	130 ± 118
42	NH			Me	33	-	-
43	NH		Me		33	168 ± 73	-
44	NH	Me			31	112 ± 26	-
45	NH		*cy*-pentyl		59	102 ± 19	135 ± 90
46	NH			SO_2Ph	10	128 ± 54	-
47	NH			F	com.	-	-
48	NH		F		com.	-	-
49	NH	F			12	107 ± 30	-
50	NH	H	H	H	com.	-	-
51	NH			OMe	21	-	-
52	NH		OMe		37	-	-
53	NH	OMe			19	-	-
54	NH		di-OMe		71	-	-
55	NH			morpholino	10	-	-
56	NH	morpholino			25	-	-
57	NH			NO_2	10	-	37 ± 2.3
58	NH		NO_2		10	-	-
59	NH	CO_2H			com.	-	-
60	NH			CN	10	-	-
61	NH	NH_2		Cl	com.	-	-
62	NH	Me	Cl		com.	-	58 ± 20

Table 1. Summary of yields and binding affinities of flufenamic acid derivatives for BF3 site and DHT binding site (com.: commercially available; - : does not bind).

Task II. **Evaluation and Study of Synthesized Derivatives for Optimization of AR Co-Regulator Interaction Inhibitors.**

II.1. SAR studies. We studied the ability of each compound to displace co-regulators from AR and analyzed the influence of structural changes of the chemical scaffold on binding affinity using SAR modeling. As expected, ester **2** didn't show any affinity for any external binding sites (table 1). As a consequence, the free carboxylic acid moiety is considered as an essential point of guaranty to bind the BF3 pocket *via* the G724 residue (figure 1C). Moreover, replacement of the amine group either with an ether (**3**) or a thioether (**4**) linkage gave inactive compounds. Addition of a methylene (**5** and **6**) or a carbonyl (**7**) group didn't improve the affinity of the starting scaffold. Thus, the diphenylamine function could not be replaced or extended presumably due to a hydrogen bond formed with the proline residue P723 (figure 1C). Finally, tricyclic compounds (**8**, **10**, **11** and **12**) weren't able to displace the fluorescent peptide from the AF2 pocket. Tricyclic structures are scaffolds to avoid if one wants to enhance the affinity of small molecules for the BF3 binding site. After testing those individual FLF analogs, we retained the anthranilic scaffold as a main core and studied a small library with various substituents R at different positions on the second aromatic ring (table 2). The first observation that came to us was that substituents in the *ortho* position showed higher IC_{50} values compare to those in the *meta* position, and even more to those in the *para* positions. A steric effect is induced by groups in the *ortho* position, independently of their nature, and prevent from an adequate fitting of the small molecule within the binding site.

R	*o*	*m*	*p*	π
*cy*Hex			+	2.82
SPh	-		+	2.32
OPh	+	+	+	2.08
Bn	+	+	+	2.01
Ph	~	~	+	1.96
*t*Bu		-	+	1.68
OBn	~	+		1.66
*n*Hex			+	
O-*n*Hex			-	
OPyridin		-	-	
*i*Pr	-	-	-	1.53
CF_3	-	~	+	0.88
SCF_3			-	
Me	-	-	-	0.56
SO_2Ph			-	0.27
F	-	-	-	0.14
H	-	-	-	0.00
OMe	-	-	-	-0.02
Morpholino	-		-	
NO_2		-	-	-0.57
CN			-	-0.8

Table 2. Influence of the position of the substituents of the second aromatic ring on the affinity for the BF3 pocket (+ : IC_{50} < 60 µM; ~ : 60 µM < IC_{50} < 100 µM; - : IC_{50} > 100 µM).

We also noticed that the most active FLF derivatives were the ones that present an additional hydrophobic substituent. This main structural feature of these compounds is illustrated by the figure 3.When we looked at the potential influence of the electronic effect of the substituents (σ value), no correlation was found to improve the binding of the small molecule to the extern site (figure 3A). However a good correlation (figure 3B, r^2=0.72) was found between the hydrophobic character of the substituents, reflected by the π value, and the IC_{50}. Indeed the affinity of the analogs increases with their hydrophobicity and this relationship is in agreement with the general hydrophobic character of the solvent-exposed BF3 pocket. In figure 3, only *para* substituents are plotted, the groups in *ortho* position were removed according to previous evidence, to eliminate the influence of a steric effect.

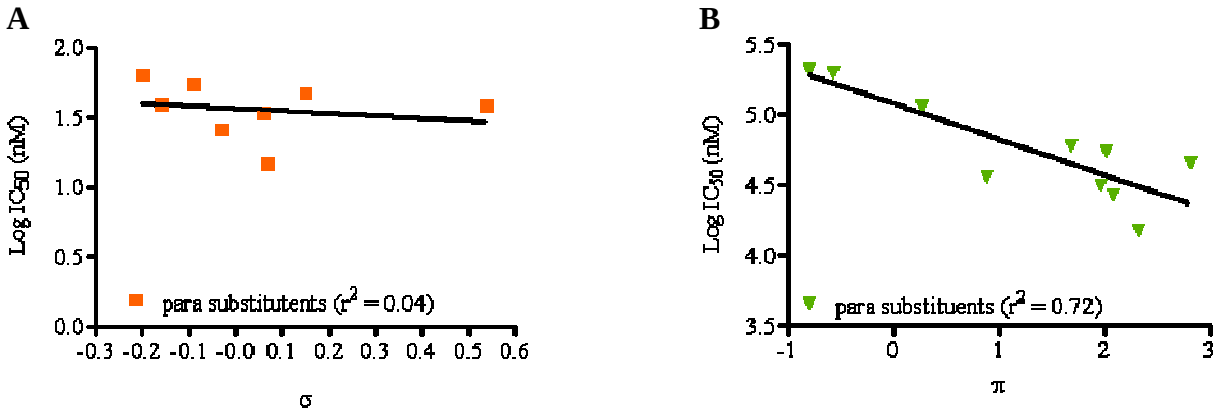


Figure 3. Relationships between electronic effect (A) or hydrophobicity (B) of the *para* substituents on the second aromatic ring and binding constants measured in a FP competition assay for FLF analogs.

From this series of analogs we also noticed that an expansion of the core scaffold is possible. As indicated in table 1, the introduction of a third (**14**-**26**) and even a fourth six-membered ring (**16**) didn't alter the binding to the external site. Conversely, heteroatom linkages, like thiophenoxy (**14**) or phenoxy groups (**17**), were more tolerated than hydrophobic groups, such as benzyl (**20**) or phenyl (**23**) groups.
Therefore the derivatives **14** and **16** were considered as the two best inhibitors of this focused library as they showed the highest ability to compete with the CoA peptide in the low micromolar range. Analogs **14** and **16** are considered as lead compounds and future structure optimizations will correlate their features to design new scaffolds. Confirmation of these binding affinities remains to be done via another biochemical assay (alpha-screen assay, TR, FRET, TR-FRET…) that will allow us to measure more precisely binding constants in the nanomolar range.

II.1. Secondary Biochemical Assays. *Binding affinity for the hormone binding site.* We investigated the FLF derivatives ability to displace the dihydrotestosterone from the ligand binding domain. Indeed, if the small molecule competes with the natural ligand then it will result as a false positive in the FP CoR competition assay and a wrong mode of action will be assigned to the drug. Screening a quite large set of small molecules requires a standardized and automated binding assay. Many current assays use fluorescently labeled ligands which are significantly different from the native ligand whereas radioactive labeled ligands are considered as more reliable probes by their structural similarity with the natural ligand. To our best knowledge only a few published reports have described a radiometric AR ligand binding assay with potential application to HTS campaign [8-11]. Historically, biochemical assays have been limited to low throughput due to the lack of necessary amounts of pure and functional AR protein. Purification of AR is complicated due to low solubility, instability in the absence of ligand, and problems of aggregation [12]. Most common methods use commercially available unliganded AR which is very expensive and delicate to handle. For those reasons, we decided to express His_6-AR-LBD in *E. coli* and to purify to homogeneity in the presence of DHT using a modified version of published protocols [13] (see supporting data). We attempted to develop a robust competition assay using our home-made DHT-liganded AR, ^{3}H-DHT and make it applicable to HTS purposes.

We first chose a radiometric ELISA-like assay using classic functionalized beads to capture the His_6-tagged AR and requiring filtration steps. We rapidly realized that the filtration steps would be a safety issue handling radioactive solutions along a HTS process. Scintillation proximity assay (SPA) technology, characterized by its sensitivity, and reliability, and the fact that no separation step is required, has become a very important technique to perform these screens in HTS format. This "mix-and-read" technique involves a fluoroscintillant coated support (beads, plate) that is functionalized (e.i. Ni chelate function) to capture one of the assay partners. When the radioligand (^{3}H-DHT) binds to the trapped protein (His_6-AR-LBD), it is in turn close enough to the fluoroscintillant coating to allow energy transfer from β-particles, resulting in photon emission. In a first attempt, SPA beads were employed to support the AR. The AR on beads solution was dispensed in microplates before adding the small molecule in competition with the ^{3}H-DHT. We couldn't obtain a satisfying method to dispense a homogeneous bead solution into each well as it resulted in less reproducible measurements with high standard deviations. Finally, we used commercially available 384 well FlashPlates® precoated with a Ni-chelate as a SPA support. We developed an AR competition ligand binding assay using SPA 384 well plates and liganded AR-LBD protein expressed in *E. coli*. This standardized and highly reproducible assay can be easily automated for HTS. The radiolabeled ligand is added at the very last step to reduce handling of radioactive material and to avoid safety problems. In addition to AR we show that this assay can be used to measure ligand affinities for different nuclear receptors like the peroxisome proliferation activated receptor (PPARγ) and the thyroid receptor (TR) (manuscript submitted to JBS, see Reportable Outcomes).

This optimized SPA competition assay allowed us to study the displacement of ^{3}H-DHT by small molecules present at various concentrations, establish dose-response curve analysis, and determine IC_{50} values (table 1). In this particular assay, DHT showed a Kd = 61 nM ± 18. In general, FLF analogs didn't displace DHT or did with a negligible binding effect showing IC_{50} values 100 fold superior to the natural ligand. Interestingly, the

n-hexyloxy derivative **32** was found to bind to the DHT site at a low micromolar range whereas no activity was observed in the SRC2-3 competition assay. The compound **32** was tested with a fluorescent probe in a commercialized FP assay (PolarScreen Androgen Receptor Competitor Assay Kit, Green, Invitrogen) that confirmed a binding constant of 0.6 µM ± 0.3 for the DHT site. This suggests that **32** could have the profile of an AR agonist that could displace DHT without disrupting the recruitment of transcriptional CoR.

Considering the lead compounds, we could notice that **14** had a 20 time tighter binding constant (3 µM ± 0.2) for the DHT binding site than **16** (57 µM ± 54) whereas they showed similar affinities for the CoR pocket. One can imagine that monosubstituted compounds may fit better into the DHT binding site than disubstituted ones. This difference in activity made the derivative **16** a more specific inhibitor and we used it as a reference compound for the following assays.

II.2. Transcriptional Activation Activity. We are in the process of evaluating the bioactivity of the synthesized FLF derivatives on AR signaling in cellular systems. Each compound will be evaluated regarding its ability to reduce AR transcription activity. For that matter we use MDA-kb2 cell line, human breast cancer cells that possess an endogenous human AR and MMTV-neo-luciferase gene construct and elicit an increase in luciferase activity in the presence of an androgen [14]. Luciferase production is detected by its activity via a commercially available luminescence assay (Bright-Glo™, Promega). Reading of luminescence of the produced oxyluciferin reflects the luciferase activity and so the transcription activity of the AR. The best response was obtained at a cell density of 300,000 cells/ mL (data not shown) in 96 well microplates. We studied the dose response of DHT alone or in the presence of drugs after letting the cells attach during different time periods and after different incubation periods in presence of drug at 37ºC (figure 4).

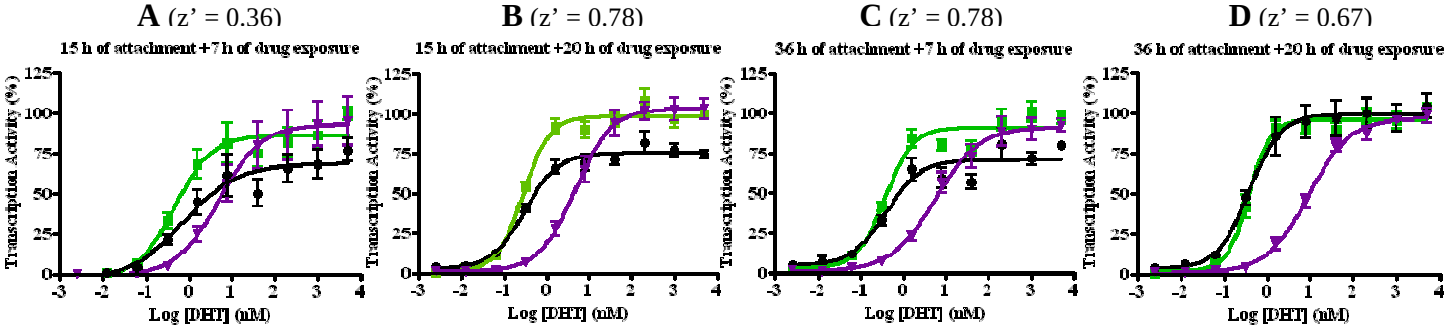


Figure 4. Dose responses of dihydrotestosterone (DHT) alone (■), with compound **16** at 50 µM (●) or with hydroxyflutamide (OHF) at 1 µM (▼). Luminescence activity is read on an EnVision (PerkinElmer) in 96 well microplates, at 300 000 cells/mL, at room temperature. Data is normalized from four independent experiments.

When we let the cells attach for 15hours, we observed that AR transcription activity in MDA-kb2 is not optimal after 7 hours of drug exposure (figure 4A). High standard deviations over the triplicates are obtained and z' value is too low ($0.5 < z' < 1$). Cells started to be responsive after drug exposure of 20 hours (figure 4B) and the assay gave robust measurements (z' = 0.78). We could already notice that compound **16** had an influence on AR transcription activity. On the other hand, when the cells are let attach for 36h, 7 hours in presence of drugs seemed to be enough to get satisfying dose responses (figure 4C, z' = 0.78). In the situation 4D, the effect of compound **16** is no longer observed. The compound itself may be degraded by this time or the response attenuated. For practical reasons, the conditions 4B were preferred (15 hours of attachment, 20 hours of drug exposure) to conditions 4C (36 hours of attachment, 7 hours of drug exposure).

DHT was also dosed in presence of 1 µM of hydroxyflutamide (OHF), a known competitor of DHT [15-17]. In this case, we could observe a shift in IC_{50} suggesting that both DHT and OHF were competing for the same binding site. Conversely, when DHT and analog **16** are added to the cells, DHT didn't seem to be displaced (no

shift in IC_{50}) but the AR transcription activity is significantly reduced (25%). This indicates that the FLF derivative has the profile of a partial antagonist that acts through another path than anti-hormones like OHF.

Then we focused our efforts on fixing the DHT optimal concentration to reach the highest amplitude of signal (figure 5A, top value - bottom value) while dosing a tested inhibitor. OHF and compound **16** were dosed response in the presence of different concentrations of DHT (figure 5A). The figure 4B illustrates the variation of the signal amplitude for dosages of compound **16** at five different DHT concentrations at four different cell passages. We could observe that for low (0.3 nM) or high (200 nM) DHT concentrations dose responses for **16** were almost flat, whereas in a 5-10 nM DHT range we were able to obtain workable curves. The plot 5B uses a 2^{nd} order polynomial equation to fit variation points of each experiment and allows us to estimate the optimal DHT concentration as the maximum of each curve. Taking the average of the four maxima, the optimized DHT concentration appeared to be 8 nM (dotted line on figure 5B) and will be fixed for the screening of all synthesized compounds.

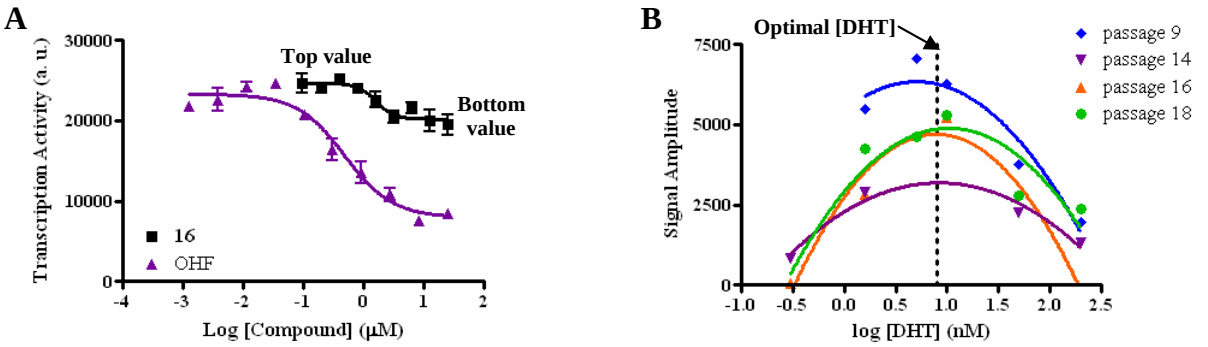


Figure 5. Optimization of DHT concentration in a luciferase reporter transcription assay in MDA-kb2. **A.** Dose responses of compound **16** and hydroxyflutamide (OHF) in the presence of 0.3 nM DHT. Signal amplitude of one dose response is calculated from the top and bottom values of the curves. **B.** Influence of DHT concentration on the signal amplitude for compound **16** dosages at different cell passages. Optimal DHT concentration is obtained on maxima average.

The above optimizations of this transcription assay were performed in 96 well microplates. For HTS purpose, we tried to adapt those optimized conditions in a 384 well assay format. Unfortunately, our diverse attempts never succeeded to obtain reproducible results. We couldn't obtain a satisfying uniformity of signal across the microplate according to NIH guidelines [18]. The synthesized FLF derivatives will then be tested in a 96 well format.

Depending on the results of the complete screen of all compounds regarding their transcription activity, we will study the native transcriptional response in LNCaP adenocarcinoma cell line in presence of potentials inhibitors by using quantitative real time PCR.

Specificity studies. *Cross-testing with other nuclear receptors.* In order to confirm that those transcription inhibitors were specifically binding to AR and didn't interfere with the activity of other receptors, we tested them towards TRα, TRβ and PPARγ. FP competition assays were conducted using a fluorescently labeled peptide based on a similar procedure as described for AR [2] (see I.4.). Each compound was incubated at a unique final concentration of 25 µM (data not shown). None of them were able to displace the CoA peptide bound to the various tested nuclear receptors.

Additionally, a transcription assay with the MDA-kb2 cell line, which expresses the glucocorticoid receptor (GR) and contains glucorticoid response elements, allowed us to check the potential affinity of our compounds

for GR. Figure 6 gathers four normalized independent experiments and robustly illustrates that **16** didn't compete with the synthetic GR ligand, medroxyprogesterone acetate (MPA).

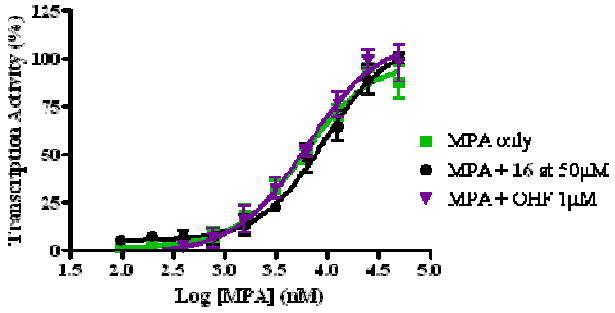


Figure 6. Dose responses of medroxyprogesterone acetate (MPA) alone, in presence of compound **16** at 50 µM and in presence of hydroxyflutamide (OHF) at 1 µM. Data shown is normalized from four independent experiments.

In general, FLF derivatives didn't show any affinity for other nuclear receptors and consequently appeared to be very specific inhibitors of AR transcription activity.

We evaluated for each compound its ADMET profile in cells by determining the aqueous solubility, the absorption profile into, and the toxicity profile in cells.

II.3. Early Pharmacology characterization. *Solubility Evaluation.* The solubility of each FLF analog was determined in PBS buffer containing 5% DMSO, reflecting the liquid conditions of the FP and SPA assays. This assay was carried out by allowing equilibrium solubility to establish and separating insoluble material by filtration following Millipore guidelines [19]. The UV absorption was measured at 350nm, subtracted from background (DMSO) and limit of aqueous solubility was determined (figure 7).

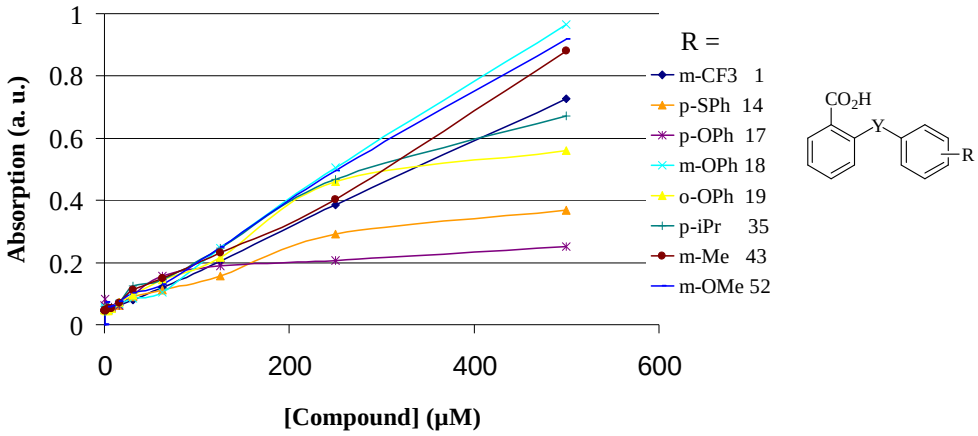


Figure 7. Solubility profiles evaluated by UV absorption for a set of flufenamic acid derivatives.

For compounds **14**, **17**, and **19** a plateau in absorption was reached for concentrations higher than 250 µM meaning that those compounds can easily be solubilized in the assay media up to 250 µM. Overall, the derivatives were soluble up to 125 µM; consequently, no solubility issues interfered with measurements of the binding affinities.

II.3. Early Pharmacology characterization. *Permeability Profiles.* We measured the permeability of each synthesized compound and their potential retention in the lipid layer using a parallel artificial membrane permeation assay (PAMPA) [20]. The partition of the derivatives between a donor well and acceptor well separated by a lipid layer was measured by UV-absorption. The assay was carried out at neutral pH imitating intravenous conditions. Standards used were Verapamil ($P_e = 1505.10^{-6}$ cm/s) as a high permeability standard, Carbamazepine ($P_e = 150.10^{-6}$cm/s) as a medium permeability standard, and Ranitidine ($P_e = 2.3.10^{-6}$ cm/s) as a low permeability standard (standards are represented by colored clusters in figure 8A). Although FLF analogs were highly soluble in buffer, we observed that they exhibited medium ($188 > P_e > 50. 10^{-6}$cm/s) to very poor ($P_e < 50. 10^{-6}$cm/s) cell permeability.

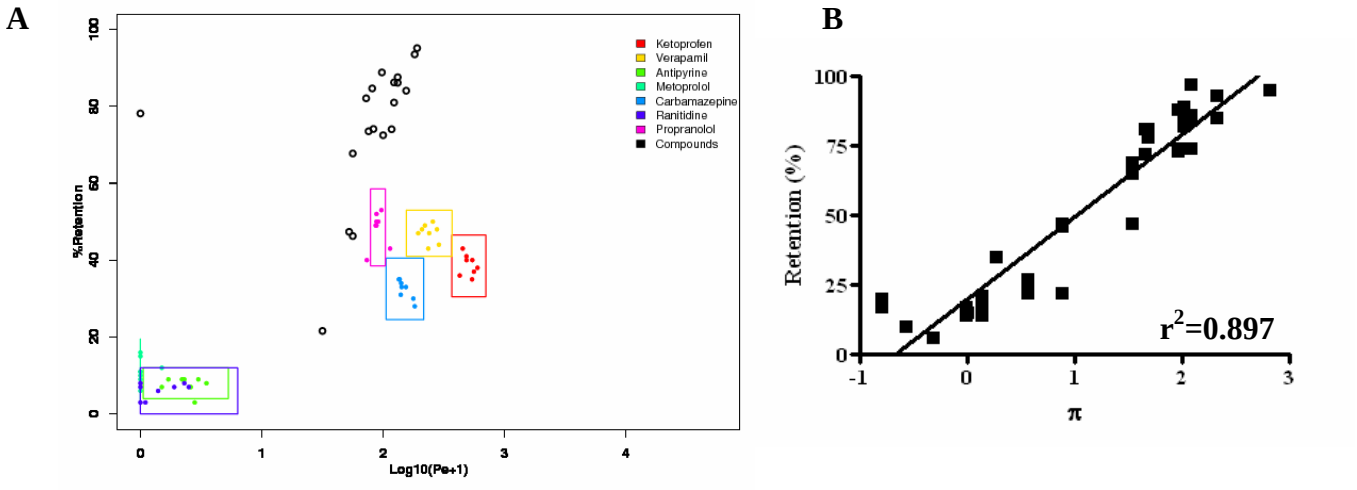


Figure 8. A. Scatter plot of compound retention (%) in the lipid layer versus permeability across the cell membrane measured with a PAMPA assay. A set of compounds is plotted (**o**) as well as standard references (colored dots). **B.** Scatter plot of membrane retention versus π, hydrophobic character, of each compound.

As illustrated in figure 8B, the more hydrophobic the compounds were the more they accumulated in the lipid layer ($r^2 = 0.897$). FLF analogs showed high membrane retention rates but their ability to diffuse across the lipid layer was very weak. Since those small molecules have a strong affinity for the cell membrane, we logically expect efficacy constants measured in cell based transcription assays to be limited by this unfavorable permeability factor.

II.3. Early Pharmacology characterization. *Cytotoxicity.* Finally, we checked the cytotoxicity of all derivatives in prostate cancer androgen-sensitive (LNCaP), androgen independent (PC3), liver (HepG2), lymphocyte (Raji), and kidney (Hek293) cell lines. To validate our transcription assay, we also tested our library of analogs in MDA-kb2 cell line. Each compound was incubated at 50 µM (figure 9) and at 1 µM (data not shown) with cultured cells to determine the cell viability after 72 hour of drug exposure. The cytotoxicity was evaluated by using the Alamar Blue Assay [21] (Biosource / Invitrogen) and reading fluorescence. Figure 9 gathers all data observed at 50 µM for the various cell lines.

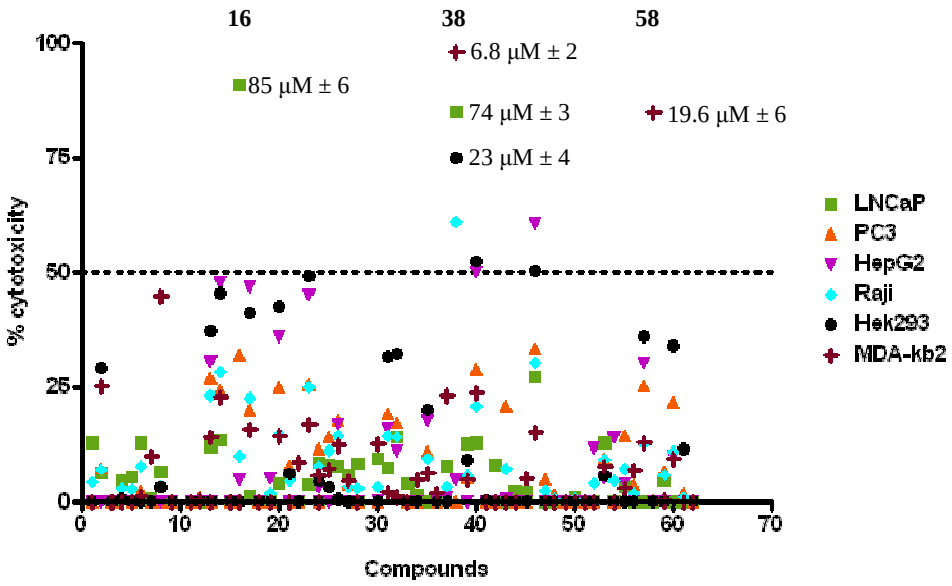


Figure 9. Cytotoxicity studies in various cell lines after 72 hour exposure of each drug at 50 µM. The most cytotoxic small molecules were assayed in a dose response manner, IC_{50} values are indicated close to the corresponding point.

Overall the small molecules didn't show any particular cytotoxicity at this high concentration independently of the nature of the cell line. However, we could observe the cytotoxicity of compounds **16** and **38** in LNCaP, an androgen-responsive prostate cancer cell line. These derivatives were assayed in a dose response manner and IC_{50}s were calculated. Moreover, we could confirm that only two compounds were toxic for the MDA-kb2 cell line and that no major cytotoxic effect could interfere during the transcription assay described above.

Key Research Accomplishments

- ✓ Design and synthesis of 80 novel flufenamic acid derivatives
- ✓ Full structural characterization and quality control for each compound
- ✓ Determination of binding affinities for each compound
- ✓ Establishment of SAR models to optimize the structure of the future scaffolds
- ✓ Development of a novel HTS SPA ligand binding assay for the Androgen Receptor and applicable to other nuclear receptors
- ✓ Validation of the specific binding of the flufenamic acid derivatives to the external activation site of the Androgen Receptor (no displacement of the natural ligand, DHT)
- ✓ Optimization of a transcription assay in MDA-kb2 cell line
- ✓ Establishment of a transcription activity profile of a lead compound (**16** can be considered as a partial antagonist of CoR recruitment)
- ✓ Validation of the specific affinity for the Androgen Receptor (no affinity found for other nuclear receptors)
- ✓ Determination of solubility profile in biologic environment for each compound
- ✓ Determination of cell permeability and membrane retention for each compound
- ✓ Evaluation of the cytotoxic character of each compound in diverse cell lines

Reportable Outcomes

1. Manuscript submitted to JBS
A High-Throughput Competition Ligand Binding Assay for the Androgen Receptor and other Nuclear Receptors
Clémentine Féau, Leggy A. Arnold, Aaron Kosinski, and R. Kiplin Guy.

Abstract: Evaluating endocrine activities of environmental chemicals or screening for new small molecule inhibitors of nuclear receptor-ligand interactions requires standardized and automated binding assays. Many current assays rely on fluorescently labeled ligands which are significantly different from the native ligand. We describe an androgen receptor (AR) competition binding scintillation proximity assay using Ni-coated 384 well FlashPlates® and liganded AR-LBD protein. This highly reproducible and low cost assay can be easily automated for HTS. Additionally, we show that this assay can be used to measure ligand affinities for a range of nuclear receptors (peroxisome proliferation activated receptor γ, thyroid receptor α and β).

2. Training on Molecular Operating Environment (MOE), November 2007, St Jude Children's Research Hospital
- _Overview of the software
- _Pharmacophore generation and modeling studies
- _Cheminformatics and Quantitative Structure Activity Relationship

3. Training on DiscoveryGate, September 2007, St Jude Children's Research Hospital
- _Overview of the software
- _Use of the database

4. Poster presentation, June 2007, Combinatorial Chemistry Gordon Conference, Cambridge (New Hampshire, USA)
Small Molecule Inhibitors of the Androgen Receptor Transcriptional Activity as Novel Targets in Prostate Cancer Drug Discovery

5. Poster presentation, March 2007, Keystone Symposia, Steamboat Springs (Colorado, USA)
Androgen Receptor/ Transcriptional Coregulator Interactions as Novel Targets in Prostate Cancer Drug Discovery

Abstract: Androgens, mediated by the Androgen Receptor (AR), play a crucial role in prostate cancer. Current treatments include antiandrogenic drugs competing with natural androgens and antagonize the transcriptional activity of the AR. Although widely used, these drugs have shown significant side effects and in addition tumors have become resistant suggesting mutations of the receptor.
Regulation of gene expression by AR requires the binding to its natural ligand and assembly of a dynamic multi-protein complex including obligate coregulatory proteins (CoR). The blockage of the interaction between liganded AR and CoR by small molecules has shown to inhibit gene transcription. These compounds represent a new class of drugs and might overcome tumor resistance during prostate cancer treatment.
Preliminary data revealed that the non-steroidal anti-inflammatory drugs, like flufenamic acid, bind to AR and inhibit the recruitment of CoR. Herein we describe the development of small molecules, analogs of flufenamic acid, using biochemical and cellular assays to establish careful structure activity relationship (SAR) models.
Small molecule inhibitors of the interaction between liganded AR and CoR represent powerful assets to study the mechanism of AR transcriptional function and a new potential therapeutic modality for prostate cancer.

Conclusion

During this first year of funding, we were able to take over the primary results of a HTS campaign providing a hit compound, flufenamic acid (FLF), potential inhibitor of the interactions between transcriptional co-regulators (CoR) and the Androgen Receptor (AR). We designed, synthesized and fully characterized diverse analogs of FLF. We tested their ability to displace CoR with a FP competition binding assay and established SAR models based on these results. We concluded that the carboxylic acid and the amine moieties were crucial for the mode of binding of the molecule. To fit the CoR pocket, the small molecules had to be flexible and may contain up to four aromatic rings. The addition of hydrophobic substituents to the initial scaffold contributed to increase affinity of the compound for the external binding site. After this first round of optimization of the FLF scaffold, the novel analogs showed an enhancement of 10 fold in binding affinity. However, this improvement creates a need for us to develop a more precise biochemical assay that will allow us to test the optimized compounds in the nanomolar range.

We confirmed the specificity of binding of those new compounds for the AR. We could prove that they show none affinity for the other members of the nuclear receptor family. And regarding their mode of action, we observed that they were not binding to the ligand binding site and so could not displace DHT like the current antiandrogens. Moreover, first results of a transcription assay showed that our lead compound **16** reduced AR transcription activity by 25% acting through another binding site other than the antiandrogen, hydroxyflutamide. This behavior in cells confirmed the results from both previous competition binding assays. We plan to test the transcription activity of analog **16** in LNCaP in the presence of high concentrations of DHT to confirm whether or not it is DHT competitive. The screen of all compounds on the transcription level remains to be done and to be analyzed. Depending on the obtained results, we will eventually start a study of native transcriptional response in the LNCaP adenocarcinoma cell line in presence of potentials inhibitors by using quantitative real time PCR.

Finally we started to draw the pharmacological profile of each synthesized compound. This family of molecules isn't cytotoxic towards the different tested cell lines, except compound **16** which showed selective toxicity in LNCaP cells. This result confirms once again the previous promising activity measured in the biochemical assays. This characteristic suggests that the compounds will have good cell viability in general and allow us to test them easily in diverse cell systems. These FLF analogs were found to be highly soluble in biological media but diffused very poorly across the cellular membrane. Their hydrophobic feature enhances their affinity for the lipidic cell membrane. As a consequence, we expect potency measurements in cell based systems to be erroneous due to this limiting factor. In a next round of optimization, we plan to improve the cell permeability of the future compounds along with the scaffold extension. Based on the FLF crystal structure we can envision that four-ringed compounds may fit into the BF3 pocket. A systematic SAR of additional substituents on the third and fourth aromatic rings will lead us to key structural features that a better AR transcriptional inhibitor should bear.

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PUBLICATIONS

- **Synthesis of a Coumarin-based Europium Complex for Bioanalyte Labeling**

  C. Féau, E. Klein, P. Kerth, L. Lebeau, *Bioorg Med Chem Lett*, **2007**, 17 (6), 1499-503.

- **Synthesis of Novel Coumarins and Azacoumarins**

  C. Féau, P. Kerth, L. Lebeau, in preparation for *J Org Chem*

- ***A High-Throughput Competition Ligand Binding Assay for the Androgen Receptor and other Nuclear Receptors***

  C. Féau, L. A. Arnold, A. Kosinski, R. K. Guy, submitted to *JBS*

- **Synthesis of Small Inhibitors of the Androgen Receptor Transcriptional Activity**

  C. Féau, L. A. Arnold, A. Kosinski, R. K. Guy, in preparation for *Angew Chem Int Ed*

Supporting Data

Expression and Purification of liganded AR Protein

cAR-LBD (His_6; residues 663-919) was expressed in *E. coli* and purified to homogeneity in the presence of dihydrotestosterone (DHT) using a modified version of published protocols [13]. Briefly, (pKBU553) was transformed into OneShot BL21 Star (DE3) chemically competent *E. coli* (Invitrogen) and streaked onto a LB agar + 1 X Carbenicillin (100 µg/ml) plate. A single colony from this plate was used to inoculate a seed culture which was then grown up overnight at 37°C in a shaking incubator. The next day 2 L of 2 X LB+1X Carbenicillin and 10 µM DHT were seeded at 0.1 OD and grown at 25 °C with shaking until OD reached 0.6-0.8. Expression was induced with 60 µM isopropyl-β-D-thiogalactoside, and left to grow 14-16 h at 17 °C. Cells from a 2 L culture were pelleted by spinning for 20 min at 5000 g. The pelleted cells are then transferred into a 50 mL conical tube, flash frozen in liquid nitrogen, and stored at -80 °C. All buffers for the purification were stored on ice. Purification of the AR-LBD began by thawing the 50 ml conical tube containing the pelleted cells on ice. 30 mL of freshly prepared buffer 1 (50 mM Tris pH 7.5, 150 mM NaCl, 10 µM DHT, 0.1 mM phenylmethylsulfonyl fluoride, 10 mg/L Lysozyme, and Roche Complete EDTA free protease inhibitor cocktail tablet) was added followed by resuspension using a spatula. Sonication in ice/water was carried out in 2 minutes intervals at 30% amplitude (Branson Digital Sonifier) followed by a 2 minute break. After six or more repetitions the suspension was no longer "gooey." Insoluble cellular debris was pelleted via ultracentrifugation (2 x 30 min at 100,000 *g* at 4 °C). To a 50 ml conical tube Talon resin (1 ml per liter cell culture) was add and washed (2 * 15 ml) with freshly prepared buffer 2 (50 mM $NaPO_4$ pH 8.0, 300 mM NaCl, 10% glycerol, 0.2 mM TCEP, 0.1 mM PMSF, 2 µM DHT). The washing and elution steps were carried out by resuspending the Talon resin in the conical tube, centrifuging it for 5 min at 4°C at 50 *g*, and decanting the supernatant. The protein supernatant was added to Talon resin (40 ml of supernatant for each conical tube) and rotated gently for overnight at 4 °C. The resin was pelleted by centrifuging for 5 min followed by washing (5 * 10 ml) with buffer 3 (50 mM $NaPO_4$ pH 8.0, 300 mM NaCl, 10% glycerol, 0.2 mM TCEP, 0.1 mM PMSF, 2 µM DHT, 10 mM imidazole). Additionally, resin was washed (5 * 10 ml) with buffer 4 (50 mM $NaPO_4$ pH 8.0, 300 mM NaCl, 10% glycerol, 0.2 mM TCEP, 0.1 mM PMSF, 2 µM DHT, 10 mM imidazole, 2 mM ATP, 10 mM $MgCl_2$). Elution was carried out in fractions equal to or less then bed volume using buffer 5 (50 mM $NaPO_4$ pH 8.0, 300 mM NaCl, 10% glycerol, 0.2 mM TCEP, 0.1 mM PMSF, 2 µM DHT, 250 mM imidazole, 100 mM KCl). Protein purity (>90 %) was assessed by SDS-PAGE and analytical size exclusion FPLC. Protein concentrations were measured by Bradford and BCA protein assays. Usually 6-8 mg of protein per liter of cell culture were obtained. The protein was dialyzed overnight against buffer 6 (50 mM HEPES pH 7.2, 150 mM Li_2SO_4, 10% glycerol, 0.2 mM TCEP, 20 µM DHT) and stored at -80 °C in buffer 6.